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Roles of MSH2 and MSH6 in Cadmium-induced G2/M checkpoint arrest in Arabidopsis roots

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ABSTRACT

DNA mismatch repair (MMR) proteins have been implicated in sensing and correcting DNA damage, and in governing cell cycle progression in the presence of structurally anomalous nucleotide lesions induced by different stresses in mammalian cells. Here, Arabidopsis seedlings were grown hydroponically on 0.5×MS media containing cadmium (Cd) at 0-4.0 mg L^{-1} for 5 d. Flow cytometry results indicated that Cd stress induced a G2/M cell cycle arrest both in *MLH1*-, *MSH2*-, *MSH6*-deficient, and in WT roots, associated with marked changes of G2/M regulatory genes, including *ATM, ATR*, *SOG*1, *BRCA*1, *WEE*1, *CYCD*4;1, *MAD*2, *CDKA*;1, *CYCB*1;2 and *CYCB*1;1. However, the Cd-induced G2/M phase arrest was markedly diminished in the *MSH2*- and *MSH6*-deficient roots, while a lack of *MLH1* had no effect on Cd-induced G2 phase arrest relative to that in the wild type roots under the corresponding Cd stress. Expression of the above G2/M

regulatory genes was altered in *MLH1, MSH2* and *MSH6*-deficient roots in response to Cd treatment. Furthermore, Cd elicited endoreplication in *MSH2*- and *MSH6*-deficient roots, but not in *MLH1*-deficient Arabidopsis roots. Results suggest that *MSH2* and *MSH6* may act as direct sensors of Cd-mediated DNA damage. Taken together, we conclude that *MSH2* and *MSH6*, but not *MLH1,* components of the MMR system are involved in the G2 phase arrest and endoreplication induced by Cd stress in Arabidopsis roots.

Key words: Arabidopsis; Cd stress; Cell cycle; G2 phase arrest; DNA damage; DNA Mismatch repair

Abbreviations:

Arabidopsis	*Arabidopsis thaliana*
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
*BRCA*1	Breast cancer susceptibility1
CDKs	Cyclin-dependent kinases
*CYCB*1;1	Cyclin B1;1
DAPI	4,6-diamidino-2-phenylindole
DDR	DNA damage response
DSB	Double strand break DNA
FCM	Flow cytometry
*GR*1	Gamma response1
MAPK	Mitogen-activated protein kinase
MMR	DNA Mismatch repair

MSH2	Mutated S homologue 2
MSH6	Mutated S homologue 6
MLH1	Mutated L homologue 1
mlh1	T-DNA insertion line of MLH1 deficiency
msh2	T-DNA insertion line of MSH2 deficiency
msh6	T-DNA insertion line of MSH6 deficiency
NER	Nucleotide excision repair
PCNA	Proliferation cell nuclear antigen
qRT-PCR	Real time quantitative reverse transcript polymerase chain reaction
RAPD	Random amplified polymorphism DNA
ROS	Reactive oxygen species
ssDNA	Single strand DNA
SOG1	Suppressor of gamma response 1
TLS	Trans-lesion synthesis
WT	Wild type (Col-0) line

1. Introduction

Cd is considered to be a highly toxic, persistent and accumulative heavy metal element, and has been listed among the top ten hazardous substances by the National Toxicology Program (NTP 2004) and by the Agency for Toxic Substances and Disease Registry (http://www.atsdr.cdc/gov/cercla/07list. html). Cd exists ubiquitously in the soil and water, mainly due to anthropogenic activities such as urban traffic and industrial processes, and is then transferred to the food chain, which may lead to genotoxicity or/and cytotoxicity to an organism's cells (Filipic, 2012; Pierron et al., 2014; Zhou et al., 2015). Thus, research

into the molecular mechanisms of Cd stress has become an important topic in environmental studies (Cui et al., 2017; Pena et al., 2012; Wang et al., 2016).

It is well known that Cd, even at low concentrations, can bind directly to DNA and lead to a wide variety of DNA damage processes such as base-base mismatches, insertion/deletion loops, DNA adducts, and DNA chain cross linking and breaks (Filipic, 2012). DNA stress in eukaryotic cells induces elaborate repair mechanisms and signal transduction pathways that can cause transient arrest of the progression through the cell cycle (Hu et al., 2016; Wang et al., 2013; Xiang et al., 2017). ATM and ATR kinases act as sensors of different types of DNA stress, coordinating stress responses with cell cycle checkpoint control and repair of such lesions (Yoshioka et al.,2006; Spampinato, 2017). Cell cycle checkpoints provide the cells with sufficient time to either cope with the damaged DNA or undergo cell death. In particular, the G2/M checkpoint allows cells to repair replication errors and damage before proceeding into mitosis, thereby ensuring genomic integrity. In plant cells, key components of the G2/M checkpoint comprise WEE1, BRCA1, ATM, ATR, and SOG1 which is activated through phosphorylation via the MAPK signalling pathway (Cools and De Veylder, 2009; Opdenakker et al., 2012; Pedroza-Garcia et al., 2016; Sjogren and Larsen, 2017; Yamane et al., 2007). Subsequently, active SOG1 induces hundreds of genes controlling the DDR including cell cycle arrest, DNA repair, endocycle onset and programmed cell death. The induction of these genes (i.e. *MAD*2, *MRE*11, *CYCB*1;2, *CYCB*1;2, *BRCA*1, *CDKA*;1 and *RAD*51) and accumulation of their encoded proteins results in inhibition of CDK activity and arrest in the G2/M phase in response to various stresses tested (Carballo et al., 2006; Hu et al., 2016; Jia et al., 2016; Pelayo et al., 2001; Rounds and Larsen, 2008; Weimer et al., 2016; Yoshiyama et al., 2009; Yoshiyama, 2016). More recently, FCM analysis showed that the

DDR can delay cell cycle progression and cause endoreplication in Arabidopsis *jhs1* mutant seedlings (Jia et al., 2016; Pena et al. 2012). However, little information is available about the checkpoint response of G2 phase-related *ATM*, *ATR* and *SOG1* genes in response to Cd stress in Arabidopsis seedlings.

Among the different DNA repair pathways in both animals and plants, MMR systems are involved in a wide range of important cellular processes. These include: (1) sensing DNA damage, signaling, reacting to and repairing DNA lesions such as mispaired bases (e.g. G/T, A/G or T/C), unpaired bases, and small insertion-deletion loop-outs (IDLs; e.g. TTTT/AAA) in DNA, which arise from escaping the DNA polymerase proof-reading activity during DNA replication, 5-methylcytosine deamination and the action of chemical mutagens, (2) inhibiting recombination between divergent DNA sequences, (3) maintaining barriers against massive genetic flow, and (4) preventing productive meiosis in interspecies hybrids (Hays, 2002; Emmanuel, 2006; Cadet and Davies, 2017). Thus, MMR plays a crucial role in confirming fidelity of DNA replication, maintaining genomic stability and governing cell cycle progression in the presence of DNA damage (Campregher et al., 2008; Wu and Vasquez, 2008). Thus, a fully functional MMR machinery can modulate prolonged G2/M phase arrest by up-regulation of G2/M regulatory proteins (i.e. Cyclin B1, Cdc2/p-Cdc2, and Cdc25C/p-Cdc25C) and/or by activating the p53, ATM and ATR signaling pathways in human cells under exogenous and endogenous stresses (Wang et al., 2013; Yan et al., 2003). In contrast, MMR deficient human cell lines are resistant to alkylating agents and bypass the G2/M arrest, indicating that the MMR has a role in post-replication checkpoints (O'Brien and Robert Brown, 2006). Pabla et al. (2011) demonstrated that MLH1, MSH6 and MSH2 are the main MMR proteins in human cells, and can play differential roles in G2 phase arrest following DNA damage

under different stresses. For example, MNNG (N-methyl-N-nitro-N-nitrosoguanidine)- and ST (Sterigmatocystin)-induced G2/M phase arrest requires hMLH1 in animal cells (O'Brien and Brown 2006; Wang et al., 2013). In contrast, nitric oxide (NO) and H_2O_2 are capable of arresting G2/M phase in hMLH1 mutant cells (Chang et al., 2003; Hofseth et al., 2003). However, IR (ionizing radiation)- and neutrophil-induced G2 phase arrest requires the MSH2 protein in primary mouse embryonic fibroblasts and colon epithelial cells (Campregher et al., 2008; Cejka et al., 2003; Marquez et al., 2003). Additionally, Yamane et al. (2007) showed that both MSH2 and MLH1 activate G2/M phase checkpoint via the BRCA1-ATR-Chk1 signaling pathway in human HCC1937 lines under 6-thioguanine (6-TG) stress. Recently, two models have been suggested to explain how the DNA damage recognized by MMR proteins can lead to cell cycle checkpoint activation. Firstly, the "futile repair cycle model" proposes that the MMR system plays an indirect role by initiating futile cycles of DNA repair, in which DNA breaks and gaps are continuously produced, ultimately causing the production of secondary lesions. In contrast, the "general DNA damage sensor model" proposes that MMR proteins may trigger stress signaling directly, leading to the induction of cell cycle arrest (Pabla et al., 2011; Wang et al., 2013). In Arabidopsis, there is little information on whether MLH1, MSH2 and MSH6 initiate G2 phase arrest of cell cycle progression in response to Cd stress. Thus, it is important to evaluate the putative roles of different MMR proteins in Cd-induced DNA damage and cell cycle arrest in Arabidopsis cells.

The principal objectives of the current study were to (1) measure cell cycle progression in response to Cd in Arabidopsis seedlings comparing WT with *mlh1*, *msh2* and *msh6* mutants; (2) determine the expression levels of DNA damage and G2M-phase-related genes, such as *ATR*, *ATM*, *SOG*1, *CYCB*1;1, *CDKA*;1, *WEE*1, by qRT-PCR analysis in the

above Arabidopsis seedlings under Cd stress, and (3) evaluate the potential roles of MMR genes *MLH*1, *MSH*2 and *MSH*6 in G2/M phase arrest and endoreplication in Arabidopsis under Cd stress.

2. Materials and methods

2.1. Plant materials, growth and treatment conditions

Arabidopsis thaliana (Arabidopsis) plants used in this study were of the Columbia ecotype WT (Col-0) and of the *mlh1*, *msh2* and *msh6* mutants. T-DNA insertion mutant lines of *msh2* (SALK_002708), *msh6* (SALK_089638), and *mlh1* (SALK_123174C) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA), and the background of the three mutants is from Col-0. The above seeds were surface-sterilized using bleach solution (1:10 dilution of hypochlorite) and ethanol mix (ethanol: water: bleach 7:2:1) at about 20 °C for 5 min, respectively, and were rinsed in sterile distilled water five times and imbibed in sterile-water for 2-4 days at 4 °C to obtain homogeneous germination (Pedroza-Garcia et al., 2016). The seeds were then sown in sterile flasks containing 150 mL of commercially available 0.5×Murashige and Skoog (MS) liquid medium (Basal Salt Mixture, Caisson, USA) with 0.5% (w/v) sucrose (pH 5.8), and supplemented with Cd at a final concentration of 0 (the control), 1.25, 2.5, and 4.0 mg L^{-1} in the form of $CdCl_2$ $2H_2O$ of analytical grade with purity 99.5% (PR China). Each flask with 20-30 plantlets was placed on a rotary shaker at about 50 rpm in an incubator (12 h light of approximately 3000 lx and 12 h dark at 21 ± 0.5 °C) for 5 d following germination. All treatments and analyses were repeated in three independent replicates.

179 *2.2. RNA extraction and qRT-PCR analysis*

180

181 For both the control and Cd treatments, about 100 mg of fresh roots were collected at 5
182 d following germination in the growth chamber, and flash frozen in liquid nitrogen prior to
183 storage at –80℃. Total RNAs were extracted and purified using RNA isolation and clean
184 up kits (EZ-10 DNAaway RNA Mini-prep Kit, Sagon). First-strand cDNA was synthesized
185 from 2 μg of total RNA using the PrimeScript^{TM} 1st strand cDNA Synthesis Kit (TaKaRa)
186 following the manufacturer's protocols. qRT-PCR analysis was carried out using 20 μL
187 reaction mixtures containing 0.4 μL of template cDNA, 0.5 μM of corresponding forward
188 and reverse primers and 10 μL 2×SYBR Mix (SYBR R Premix Ex Taq^{TM} Ⅱ (Tli RNaseH
189 Plus, TaKaRa). Reactions were run and analyzed on an iCycler iQ (Bio-Rad) according to
190 the manufacturer's instructions. PCR products were run on a 2% (w/v) agarose gel to
191 confirm the size of the amplification products and to verify the presence of a unique PCR
192 product. The specificity of amplification products was determined by melting curves, and
193 the gene expression level was normalized to that of the reference genes, ACT2 or UBQ10
194 (Konishi and Yanagisawa, 2011). IQ5 relative quantifiation software (Bio-Rad)
195 automatically calculates relative expression level of the selected genes with algorithms
196 based on the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). All analyses were repeated at
197 least three times. The primer pairs used for qRT-PCR are listed in supplemental (Sup)
198 Table S1. For detailed descriptions of expression of *SOG*1 and the other genes, see
199 Sjogren et al. (2015) and Cui et al. (2017), respectively.

200

201 *2.3. FCM analysis of cell cycle progression in roots of Arabidopsis*

202

203 To study the ploidy level of the WT and three mutant plantlets, approximately 0.1 g of

fresh roots were excised and chopped in ice cold chopping buffer (Partec, Germany) with a single-edged razor blade in a glass Petri dish (diameter, 5 cm). After 5-10 minutes, crude samples, consisting of finely minced tissue fragments, were put through two nylon filters (pore size, 50 and 30 µm) to remove cell debris. The nuclei in the filtrate were stained with DAPI (Partec, Germany) following the manufacturer's instructions. After 15 to 30 minutes at about 25 °C in the dark, the stained nuclei of the control and Cd-treated samples were analyzed using a CyFlow flow cytometer (Partec, Germany) equipped with a 365 nm laser. Fluorescence intensity was analyzed for $\geq$ 5000 nuclei, and four independent replicates were performed for each sample. Gates (Sup-Fig. S2) were determined empirically on nuclei extracted from the roots of the 5-day-old plantlets with Flowjo 10 win 64 software (BD Biosciences, San Jose, CA). Ploidy distribution calculated proportions of 2C, 4C, 8C and 16C nuclei (i.e. 100% in total for each treatment; Sup-Fig. S2).

2.4. DNA extraction and RAPD analysis

Fresh roots (about 100 mg) were collected as for the RNA extraction. Total genomic DNA was extracted and RAPD analysis was performed using 2 primers (Primers 3 and 11) screened from 12 random primers as previously described (Liu et al., 2005; Sup-Table S2). PCRs were performed, and polymorphism frequency of RAPDs, assessed by PAGE gel electrophoresis, was calculated according to Wang et al. (2016).

2.5. Statistical analysis

nnnSPSS for Windows (version 23.0) was used for statistical analysis of the results. Data are expressed as the means ± standard deviation (SD). Statistical differences among the

control and treatments were calculated using 1-way analysis of variance (ANOVA), taking $P < 0.05$ as significantly different according to the least significant differences (LSDs) tests corrected for the number of comparisons.

3. Results

3.1. Cd stress affected root growth of Arabidopsis plantlets

Exposure to Cd (1.25 - 4.0 mg•L^{-1}) for 5 d had no obvious effect on the germination rate of WT Arabidopsis seedlings compared to the control ($P < 0.05$; Table 1). Likewise, there were no statistically significant differences for fresh weight between the control and Cd-treated plantlets ($P < 0.05$) with the exception that seedlings treated with 4.0 mg•L^{-1} Cd indicated a notable decrease of fresh weight. However, a significant inverted U-shaped relationship was seen between root length and Cd level, with a correlation coefficient (r^2) of 0.939 using regression way (Table 1; Sup-Fig. S1).

Table 1

Effect of Cd on germination, fresh weight and root growth of Arabidopsis seedlings for 5 d.

Lines of Arabidopsis	Cd concentration/ mg·L^{-1}	Germination percentage/ %	Fresh weight of plantlet $^{-1}$/mg	Root growth	
				Root length/cm	Suppression rate/ %
WT	0	95.1±2.1	10.31±0.39	1.20±0.05	0.00
	1.25	95.8±1.9	11.04±0.42	1.32±0.04*	-10.00
	2.5	96.1±3.3	10.14±0.48	0.81±0.05*	32.50
	4.0	95.3±1.7	7.78±0.42*	0.52±0.09*	56.67
mlh1	0	95.6±3.2	10.32±0.24	1.22±0.04	0.00
	1.25	94.9±1.3	10.13±0.75	1.09±0.08	10.66
	2.5	95.2±3.5	9.08±0.78	0.88±0.02*	27.87
	4.0	95.6±1.8	7.69±0.34*	0.55±0.09*	54.92
msh2	0	94.2±1.6	10.29±0.31	1.23±0.03	0.00
	1.25	95.1±2.5	10.01±0.67	1.06±0.06*	13.82
	2.5	95.3±3.2	8.51±0.56	0.62±0.07*	49.59
	4.0	96.2±6.4	7.01±0.29*	0.4±0.09*	67.48
msh6	0	95.7±1.8	10.34±0.35	1.16±0.04	0.00
	1.25	95.2±3.3	9.75±0.49	0.87±0.05*	20.91
	2.5	96.5±7.7	8.73±0.53	0.55±0.07*	50.00
	4.0	94.8±3.1	7.13±0.45*	0.35±0.02*	68.18

* Significantly statistical difference from the control, respectively ($P < 0.05$).

Under the control conditions, the WT and three mutants (*mlh1*, *msh2* and *msh6*) had similar root growth and fresh weight (Table 1). A significant negative relationship was observed for the root length between the above mutants and Cd levels of 0 and 4.0 mg•L^{-1} for 5 d, with correlation coefficients (r^2) of 0.962, 0.983 and 0.985, respectively. Interestingly, seed germination percentage, fresh weight and suppression of root growth in the WT seedlings was not different from that of MLH1-deficient seedlings under Cd stresses of 2.5 - 4.0 mg•L^{-1}. In contrast, suppression of root growth was much greater in *MSH2*- and *MSH6*-deficient seedlings with a similar reduced trend under Cd stresses of 1.25 - 4.0 mg•L^{-1}. These results indicate that *MSH2*- and *MSH6*-deficient seedlings were

more sensitive to Cd toxicity than the WT and *MLH1*-deficient seedlings in this experiment (Table 1).

3.2. Cd stress induced G2 phase arrest in the WT Arabidopsis roots

To evaluate cell cycle progression in the WT roots under Cd stress for 5 d, effect of Cd stress on cell cycle arrest was determined using FCM analysis. As shown in Fig. 1, the proportion of cells with a 2C nuclear content (G0/G1 phase) was 35.2% in the control, but Cd stress significantly decreased this proportion at 4.0 mg•L^{-1} Cd, which was 23.1%. This alteration in the 2C nuclear content was accompanied by a significant increase in the proportion of cells with a 4C nuclear content: which was 39.8% and 41.4% in roots at the highest two Cd concentrations of 2.5 and 4.0 mg•L^{-1}, respectively (Fig. 1A, Sup-Fig. S1). The FCM result suggests that Cd stress could induce G2/M phase arrest in roots of the WT plantlets.

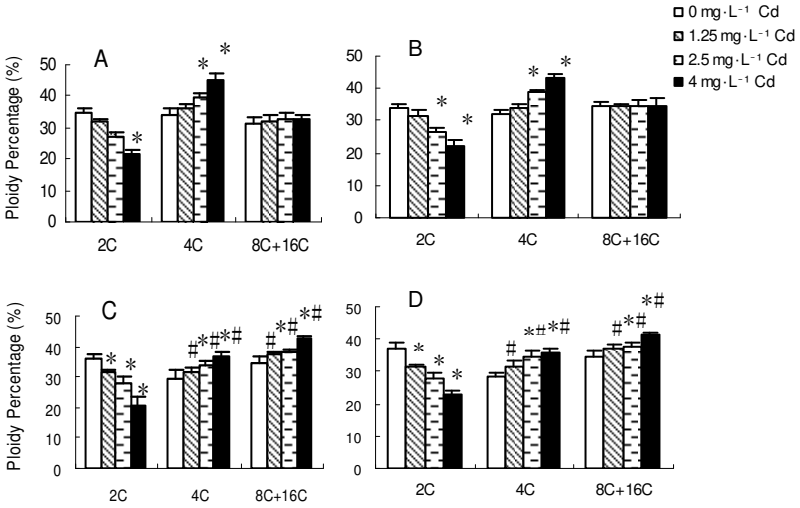

Fig.1. FCM analysis on the nuclear DNA contents of WT (A), *mlh1* (B), *msh2* (C), and *msh6* (D) in Arabidopsis roots exposed to 0 - 4.0 mg•L^{-1} Cd for 5 d. The percent distribution of cells in 2C, 4C and 8C+16C was calculated and compared with the control.

Each point represents the mean ± SD of three independent experiments. *Significantly different from the control in A-D, respectively (P<0.05), and # significantly different from the WT under the corresponding Cd stress in C-D (P < 0.05).

The effect of Cd stress on cell cycle-regulatory genes was determined by measuring the expression of marker genes for G2/M transition (*ATM* and *ATR*, *SOG*1, *WEE*1, *CYCD*4;1, *MAD*2, *CDKA*;1, *CYCB*1;2 and *CYCB*1;1) in the WT roots with and without Cd stress by qRT-PCR analysis. Two patterns of gene expression were noted. Gene expression of *ATM, ATR*, *SOG*1, *CDKA*;1, and *WEE*1 increased by 1.21- to 3.3-fold at the lowest concentration (1.25 mg•L^{-1}) of Cd, but a dose-dependent decrease was observed in expression of *CDKA*;1, and *WEE*1 with Cd concentrations above 1.25 mg•L^{-1} (Fig. 2C and 2D). The second group of cell cycle-regulatory genes, *CYCD*4;1, *MAD*2, *CYCB*1;2 and *CYCB*1;1 showed a dose-dependent reduction in the expression from 0 to 4.0 mg•L^{-1} Cd. For all these genes the maximum reduction in expression was with 4.0 mg•L^{-1} Cd with a maximum decrease of 0.12- to 0.50-fold in the expression of the *CDKA*;1, *WEE*1, *CYCD*4;1, *MAD*2, *CYCB*1;2 and *CYCB*1;1 genes relative to the control (Fig. 2C and 2D). Taken together, these findings support the hypothesis that Cd stress can mediate the aberrant expression of the above G2 phase cell cycle regulatory genes partially involved in G2/M arrest in Arabidopsis roots.

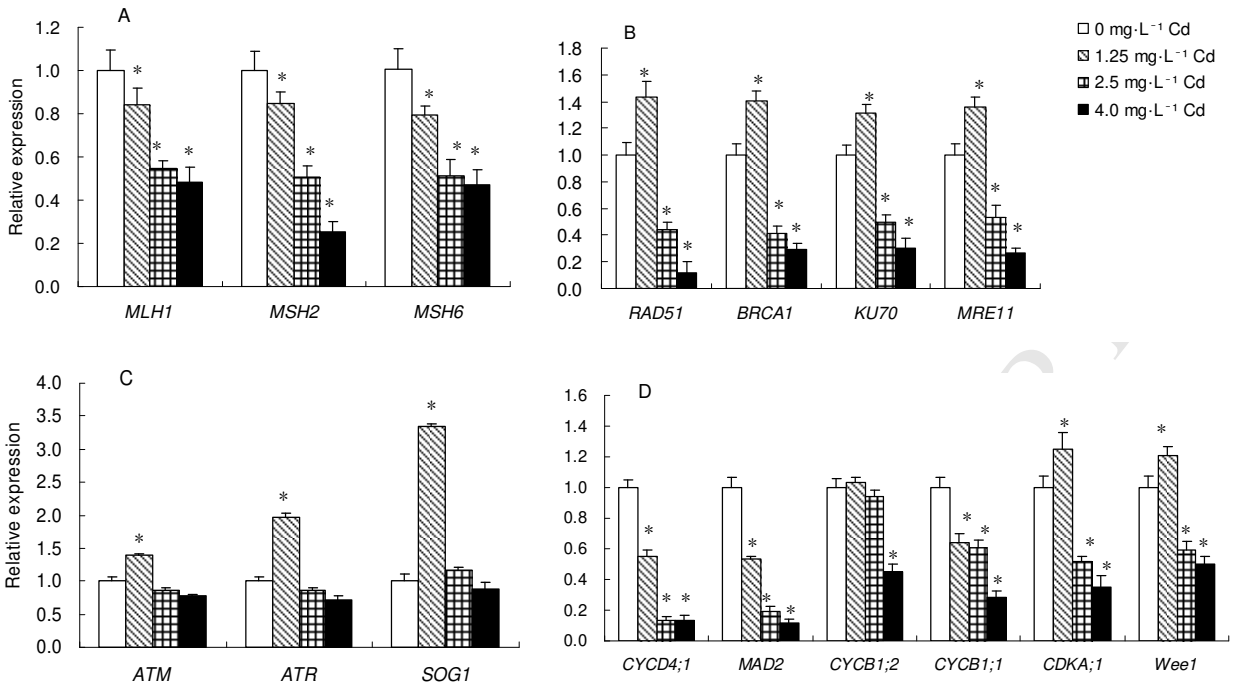

Fig. 2. Effect of Cd stress on gene expression in Arabidopsis roots for 5 d. (A) DNA mismatch repair genes *MLH*1, *MSH*2 and *MSH*6; (B) DNA damage repair genes *RAD*51, *BRCA*1, *KU*70 and *MRE*11; (C) DNA damage response genes *ATM*, *ATR* and *SOG*1; (D) G2/M marker genes *CYCB*1;1, *CDKA*;1, *WEE*1, *CYCD*4;1, *MAD*2 and *CYCB*1;2. The expression level of these genes was set to 1 in the control. Data are shown as mean ± SD by qRT-PCR. Data presented are average of three replicates. * Significantly different from the control *(P* < 0.05). House-keeping gene AtUBQ10 was used as an internal control.

3.3. Cd stress caused an MHS2- and MHS6-dependent G2/M arrest in Arabidopsis roots

FCM analyses showed that the Cd-induced G2 arrest was dramatically attenuated in the *msh*2 and *msh*6 mutants compared with the WT under the corresponding Cd stress (Fig. 1). The attenuation was of 12.7%, 14.7% and 11.6% in the *MSH2*-deficient roots, and of 10.9%, 13.2% and 15.7% in *MSH6*-deficient roots under Cd stresses of 1.25 - 4.0 mg L^{-1}, respectively. In contrast, mutation of *MLH1* had no effect on Cd-induced G2 phase

arrest. Cd stress dramatically increased the proportion of cells with 8C and 16C nuclear content, and the increase was of 6.7%, 11.3%, 15.2%, and 15.5% for MSH2-deficient roots, and of 5.7%, 11.3%, 14.31, and 12.4% for MSH6-deficient roots under 0, 1.25, 2.5 and 4.0 mg L^{-1} Cd treatments compared to WT at each Cd concentration tested, respectively. However, again there was no effect of *MLH1* mutation on the proportion of 8C and 16C nuclear content in response to Cd treatment (Fig. 1, Sup-Fig.S1). Based on the above results, we concluded that MSH2 and MSH6, but not MLH1, of the MMR system are involved in the G2 phase arrest induced by Cd stress in Arabidopsis roots.

In *MSH2*-, *MSH6*- or *MLH1*-deficient Arabidopsis roots, exposure to Cd stress strongly activated expression of *ATR*, *ATM*, *SOG*1 and *CYCB1;2* genes compared with the WT control, whereas expression of *CYCD*4;1, *RAD*51, *BRCA*1, and *MAD*2 was sharply diminished (i.e. a decrease of 0.12- to 0.23-fold) in MSH2-deficient roots under Cd stress (Fig.3). Some genes (i.e. *KU70*, *CYCB1;1*, *MRE11*) tested were down-regulated in a Cd-dependent manner in *MLH1*-deficient Arabidopsis roots. Notably, expression of *MAD*2, *MRE*11, *CYCB*1;2, *BRCA*1 and *RAD*51 genes showed obvious differences between MLH1-deficient and MSH2/MSH6-deficient roots under Cd stress. In addition, expression of *MSH*2, *MSH*6 and *MLH*1 genes was significantly suppressed in MSH2/MSH6- and MLH1-deficient roots in response to 1.25-4.0 mg L^{-1} Cd treatment compared with the WT control to some extent, respectively (Fig. 3). Taken together, the altered expression of the above genes suggests that (1) most of the genes are down-regulated in each mutant compared to the WT control, and (2) DDR is activated in Cd-stressed seedlings, which may be partially implicated in the G2 phase arrest.

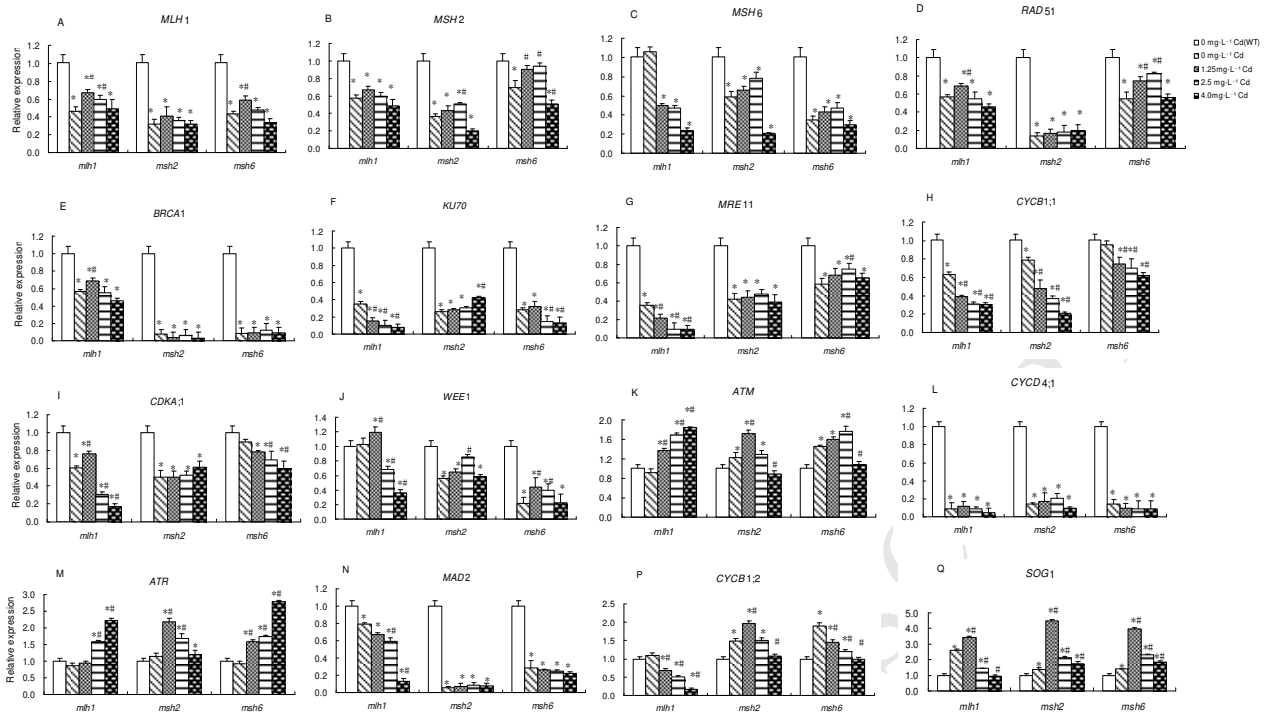

Fig. 3. Transcript expression levels in roots of *mlh1*, *msh2* and *msh6* mutants exposed to 0- 4.0 mg•L^{-1} Cd for 5 d. In A-Q, MMR genes *MLH1, MSH2* and *MSH6*; G2/M phase marker genes *CYCB*1;1, *CDKA*;1, *WEE1*, *CYCD*4;1, *MAD*2 and *CYCB*1;2; DNA damage repair genes *RAD*51, *BRCA*1, *KU7*0 and *MRE*11; and DNA damage response genes *ATM, ATR* and *SOG*1. The expression levels of the WT were set to 100% in the control by qRT-PCR analysis. Data were shown mean ± SD at least three independent experiments, and house-keeping gene AtUBQ10 was used as an internal control. * and # significantly statistical difference from the WT control and the corresponding mutant control, respectively ($P < 0.05$).

3.4. MSH2 and MSH6 may act as direct sensors of Cd-mediated DNA damage in Arabidopsis roots

To assess whether MMR proteins are acting via the futile repair cycle model or the direct DNA damage sensor signaling model in response to the Cd treatment, we examined the level of DNA damage in WT, MSH2- and MSH6-deficient roots under Cd stress using a RAPD assay. The WT and the two MMR mutants tested exhibited similar frequencies of RAPD polymorphism after Cd stress of 1.25 - 4.0 mg L^{-1} for 5 d (Fig. 4, Sup-Fig. S3). These results indicate that *MSH*2 and *MSH*6 did not lead to the formation of secondary

damage, suggesting that DNA lesions recognized by MSH2 and MSH6 could lead to G2/M cell cycle arrest through the direct signaling model in Arabidopsis roots under Cd stress.

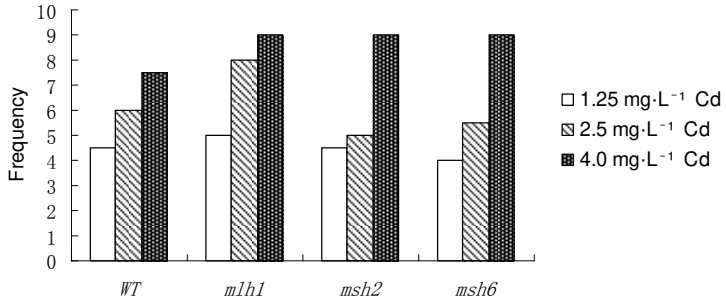

Fig. 4. RAPD polymorphism variations of Arabidopsis roots exposed to 0- 4.0 mg•L^{-1} Cd for 5 d. For all treatments, reproducible bands in at least two replicates were evaluated and calculated for polymorphism analysis.

4. Discussion

Many studies have proved that the MMR system can sense, react and repair DNA damage, thus has an utmost important role in confirming fidelity of DNA replication, in maintaining genomic stability and in governing the cell cycle progression in the presence of DNA damage induced by different stresses in mammalian cells. Such experiments indicated that MMR deficiency can lead to tumorigenesis in response to stresses through loss of cell cycle regulation and decreased apoptosis (Tsaalbi-Shtylik, 2015; Wang et al., 2013). However, little information is known about whether *MLH*1, *MSH*2 and *MSH*6 initiate G2/M phase arrest of cell cycle progression in higher plants under Cd stress. Results presented here show that Cd exposure could induce DNA damage and change remarkably gene expression of G2/M-transition-related regulation and MMR system, and thus lead to G2/M phase arrest in Arabidopsis seedlings (Figs. 1 - 2). Moreover, mutation of two MMR genes, *MSH*2 and *MSH*6 results in a significant attenuation of G2 arrest and in a marked increase of cells with 8C and 16C nuclear content compared with the WT under the corresponding Cd stress (Fig. 1), indicating that these two genes may be

important in the MMR-mediated response to Cd. Furthermore, MSH2 and MSH6 may act as direct sensors of Cd-mediated DNA damage and participate in the G2/M arrest and endoreplication under Cd stress (Figs. 1, 4). These findings provide new insights into the molecular basis of *MLH*1, *MSH*2 and *MSH*6 roles in the G2/M phase arrest caused by DNA lesions in Arabidopsis seedlings under Cd stress.

Endogenous replication stress induced by replisome factor E2F TARGET GENE1 mutant triggered a prolonged cell cycle, accompanied with a high number of the G2/M phase cells in Arabidopsis (Cools and De Veylder, 2009), while X-ray or hydroxyurea stress led to a G2/M phase arrest in root cells of onion (*Allium cepa* L.) (Pelayo et al., 2001; Carballo et al., 2006). Recently, we demonstrated that Cd stress could induce G_2 phase arrest in Arabidopsis seedlings (Cui et al., 2017). In the current study, FCM analysis indicated that Cd stress could significantly reduce the proportion of 2C cells and induce a G2/M phase arrest in the WT root cells of Arabidopsis seedlings (Fig. 1; Sup-Fig. S1). Additionally, the expression of the G_2 phase marker genes confirmed that Cd stress induced the abnormal expression of *ATM*, *ATR*, *SOG*1, *WEE*1, *CYCD*4;1, *MAD*2, *CDKA*;1, *CYCB*1;2 and *CYCB*1;1 genes (Fig. 2), which may be involved in the G2 phase arrest triggered by Cd stress in Arabidopsis roots. Although some signaling pathways, such as ATM, ATR, SOG1, WEE1, BRCA1, RAD51, CDKA;1, CYCB1;2 and CYCB1;1, have been found to be involved in the mechanism of cell cycle arrest by Cd and other stresses (Cui et al., 2017; O'Brien and Brown, 2006; Hu et al., 2016), further studies validating the exact mechanism are warranted.

A number of reports have suggested that MLH1, MSH2 and MSH6 proteins can recognize DNA damage and act as signaling mediators for activation of cellular DNA

damage responses (O'Brien and Brown, 2006; Tennen et al., 2013; Yoshioka et al., 2006). Herein, we found that Cd stress caused significant DNA damage in Arabidopsis roots (as shown by RAPD polymorphism, Fig. 4), indicating that Cd stress can produce a genotoxic effect, including DSB and ssDNA, in Arabidopsis roots. In addition, downregulated expression of *MLH*1, *MSH*2 and *MSH*6 genes occurred at the mRNA level by Cd stress at all the concentrations tested, which was obviously different from other repair genes (i.e. *KU*70, *BRCA*1, *RAD*51) (Fig. 2), showing that Cd stress was prone to impairing MMR system in response to DNA damage in Arabidopsis roots. Furthermore, knockdown studies highlighted that MSH2 and MSH6, not MLH1, caused an G2/M arrest of the cell cycle in Arabidopsis roots following Cd stress (Fig. 1), which was consistent with the activation of a post-replication DNA-damage checkpoint (Cools and De Veylder, 2009). Also, evidence for the installation of such a checkpoint, apart from the G2/M arrest (Fig. 1), includes significantly changed expression of G2/M phase and its regulation-related genes such as *ATM*, *ATR*, *SOG*1, *WEE*1, *CYCD*4;1, *MAD*2, *CDKA*;1, *CYCB*1;2 and *CYCB*1;1 in *MLH*1-, *MSH*2- or *MSH*6-defienct roots under Cd stress (Figs. 2 - 3). When the cells undergo diverse stresses, expression of ATM or/and ATR is significantly increased, which phosphorylates SOG1; subsequently, activated SOG1 and/or WEE1 could severely affect CDKA;1 to form an active complex with Cyclin B1, leading to a G2/M arrest (Hu et al., 2016; O'Brien and Brown, 2006; Weimer et al., 2016). In this study, expression of *ATM* and *ATR* was enhanced by DNA stress via cell cycle checkpoints although MutSα was uncoupling with them in MSH2- and MSH6-deficient roots, activating *SOG*1 and *WEE*1, which could cause G2/M arrest (Figs. 1 - 5). However, in the MLH1-deficient roots, ATR and ATM received signals from MutS and activated WEE1 and SOG1, leading to G2/M phase arrest (Figs. 1 - 3, 5). Indeed, expression of *MAD*2, *MRE*11, *CYCB*1;2, *BRCA*1 and *RAD*51 genes indicated obvious differences between MLH1-deficient and MSH2/MSH6-

deficient roots under Cd stress (Fig. 3). Herein, sharply repressed expression of *MAD*2 may mediate G2-M arrest through the regulation of chromatid segregation with a dual mechanism: (i) transcriptional regulation of gene expression profiling; and/or (ii) post-transcriptional ubiquitination (Sisinni et al., 2017). Also, aberrant expression of the other genes responsible for DNA repair could affect G2/M arrest probably by altering repair efficiency (Figs. 1 - 3, 5). Taken together, the above results suggest that Cd stress induced G2/M arrest, independent of *MLH*1, but dependent on *MSH*2 and *MSH*6 genes in Arabidopsis roots.

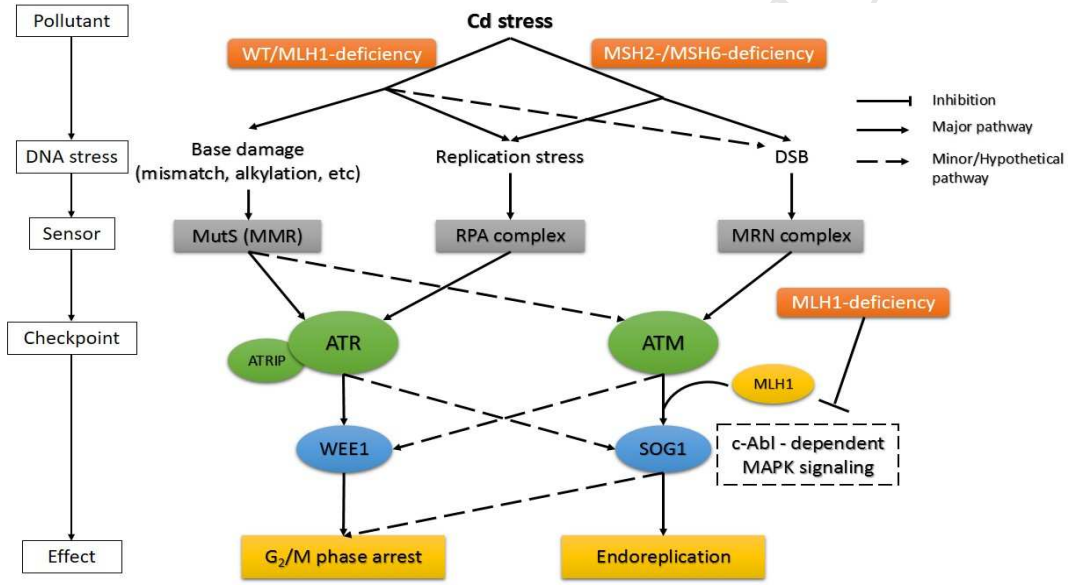

Fig. 5. Model of Cd-induced G2/M arrest and endoreplication in Arabidopsis roots. Base damage is usually major form in Cd-induced DNA stress. In WT and MLH1-deficient roots, base damage and replication stress could be sensed by MutS and RPA complex respectively, leading to activation of ATR. Then ATR activates Wee1 through phosphorylation, which can phosphorylate the inhibiting tyrosine residue of CDK in cyclin-Cdk complex, finally causing cell cycle arrest. In MSH2- and MSH6-deficient roots because of recession of sensing function caused by MutS-deficiency, more DSB are produced and sensed by MRN complex which activate ATM. ATM, as a protein kinase, has been proved that it can cross nuclei membrane through MLH1- and C-abl-dependent MAPK signaling and activates transcription factor SOG1, causing endoreplication.

Two models have been proposed to account for the reason why DNA damage

462 signaling recognized by MMR proteins may cause cell cycle checkpoint activation (Pabla
463 et al., 2011). The futile cycle model emphasizes DNA repair as the single function of MMR.
464 According to this model, a futile attempt of the MMR system to repair damaged DNA leads
465 to the generation of DNA strand breaks, as damage on the template strand is repeatedly
466 processed. However, the direct signaling model proposes two distinct functions for MMR:
467 DNA repair and DNA damage signaling. In this model, MMR proteins might directly initiate
468 DNA damage signaling that permits activation of one or more cell cycle checkpoints.
469 Indeed , these two models are not mutually exclusive, and are supported or contradicted
470 by the good experimental evidence (O'Brien and Brown, 2006; Pabla et al., 2011). Our
471 results suggest that under Cd stress, DNA damage (as evidenced by the presence of
472 RAPD polymorphism) was similar in the WT and the *msh*2/*msh*6 mutant tested after Cd
473 treatment (Fig. 4), which indicates that *MSH*2 and *MSH*6 did not lead to the formation of
474 secondary damage. Similarly, direct sensors of DNA damage signaling were recognized
475 by MLH1 and MSH2 proteins in human esophageal epithelial Het-1A cells and in mouse
476 embryonicfibroblasts, respectively (Pabla et al., 2011; Wang et al., 2013). Moreover, under
477 1.25 and 2.5 mg L^{-1} Cd stress, DNA damage was different between msh2/msh6 and *mlh1*
478 mutant (Fig. 4), suggesting functional dissociation of DNA damage repair and recognition
479 signaling. The nicks near base mismatches, O^6MeG or IDLs loci are produced during DNA
480 mismatch repair processes after replication and sensing damages (Culligan and Hays,
481 2000; Hu et al., 2016), leading to RAPD polymorphism (Fig. 4). In *mlh1* mutant, *MLH*1 and
482 many other repair genes (i.e. *KU*70, *BRCA*1, *MRE*11, *RAD*51) were prominently
483 repressed (Fig. 3) to cause low-efficient repair and long-duration of nick maintenance,
484 however, initiating MMR is significantly inhibited in msh2/msh6 mutant, which accounts for
485 differences in DNA damage between *mlh1* and msh2/msh6 mutant (Fig. 4). The above
486 observations support the direct signaling model, wherein MSH2 and MSH6 might act as

direct sensors of Cd-mediated DNA damage and be directly implicated in the initiation of DNA damage signaling responses.

The recent findings have revealed that the G2/M arrest and endoreduplication in response to different stresses are strictly dependent on MMR activity and the roles of MMR proteins in mismatch repair can be uncoupled from the MMR-dependent damage responses (Luo et al., 2004). Although MMR system repairs only DNA mismatches or mispairs in cells, it is involved in checkpoint activation in response to various forms of DNA damage (i.e. O^6MeG lesions). Moreover, while the repair can function efficiently at subnormal levels of hMLH1 or hMSH2, the checkpoint activation requires a full level of them (Luo et al., 2004; O'Brien and Brown, 2006). In the current study, the WT roots showed marked G2 phase arrest but increase growth of 10% under 1.25 mg•L^{-1} Cd stress (Table 1; Sup-Fig. S2), which suggests that the functions of MMR proteins in MMR and checkpoint signaling may involve different molecular processes (Pabla et al., 2011). The possible outcomes are complicated following MMR-dependent G2/M arrest and endoreduplication under Cd stress as follows: (1) the prolonged G2/M arrest is associated with the appearance of plantlets that display a inhibition-like phenotype such as suppressed root growth observed in both WT and mutants, mainly through ATR/ATM-Wee1 cascade (Table 1; Fig. 5); (2) some of them appear to escape from G2 arrest but undergo endoreduplication observed only in the *msh2* and *msh6* mutants, probably through TLS to bypass Cd-induced lesions in an ATM/ATR-SOG1-dependent manner (Figs. 1, 5) (Adachi et al, 2011; Hirose et al., 2003; Hu et al., 2016; Reyes et al., 2015; Tsaalbi-Shtylik et al., 2015). Alternatively, an interaction between MMR system (MSH2, MSH6 or MHL1) and DNA damage sensors/repair proteins (i.e. ATR, ATM, SOG1, MRE11, BRCA1, CYCB1;1, KU70 and MAPK) has been required for endoreduplication and for the

512 installation of cell cycle arrest as well as the co-localisation of MMR proteins, γ-H2AX foci
513 and the MRN (Mre11-Rad50- Nbs1) complex to foci of DNA damage (Hirose et al., 2003;
514 Hu et al., 2016; Manke et al., 2005; Opdenakker et al., 2012; Stojic et al，2004；Weimer et
515 al., 2016). Herein, expression of *ATM, ATR* and *SOG*1 genes was strongly activated,
516 accompanied with significantly increased proportion of cells with 8C+16C nuclear content
517 in *msh*2 or *msh*6 mutant roots under Cd stress (Figs.1-3), which accounts for
518 endoreduplication via ATM/ATR-SOG1-dependent pathway. Additionally, expression of
519 *RAD*51, *BRCA*1, and *MAD*2 genes was sharply diminished in *msh*2 mutant roots (Figs. 3,
520 5)，suggesting that these genes' expression could be mediated through a MSH2-
521 dependent pathway of ATR-SOG1 activation (Pabla et al., 2011; Sisinni et al., 2017),
522 which could similarly promote endoreduplication. However, endoreplication was not seen
523 in MLH1-deficient Arabidopsis roots under Cd stress, and many genes tested were
524 down-regulated in a Cd-dependent manner (Figs. 1, 3, 5), probably because MLH1 in
525 Arabidopsis could play an important role in c-Abl-dependent MAPK signaling just like that
526 in human/animal and then in activating SOG1 in response to DNA lesions (Kim et al.,
527 2007). Campregher et al. (2010) demonstrated that several other proteins have been
528 involved in MMR system, including clamp PCNA, DNA polymerase delta, single-strand
529 binding protein RPA, clamp loader replication factor C (RFC), exonuclease 1 (EXO1), and
530 endonuclease FEN1, which are associated with synthesizing DNA and the replication fork.
531 Thus, we could speculate that the accelerated activation of endoreplication observed
532 exclusively in the *msh*2 and *msh*6 mutants, but not in the *mlh*1 mutant, could be a
533 consequence of the interaction among RPA, MRN and MutS complex with DNA damage
534 checkpoint signaling such as ATR/ATM-Wee1 or/and ATM/ATR-SOG1 cascade under Cd
535 stress (Figs.1, 5). Taken together, exposure to Cd stress strongly activates the major
536 MSH2-ATR-Wee1 signaling cascade in WT and *mlh1* mutant, but the major MRN-ATM-

SOG1 cascade in *msh*2 and *msh6* mutants (Fig. 5).

More recently, research focus has turned to elucidating the mechanisms that regulate the dephosphorylation of checkpoint proteins, and to revealing phosphatases that govern SOG1 and other checkpoint proteins implicated in checkpoint resolution and mitotic progression (Friedhoff et al., 2016). Moreover, many results point to ATR/ATM/SOG1 as master regulators of checkpoint maintenance and resolution, and subsequent mitotic exit in plants (O'Brienand and Brown, 2006; Sjogren et al., 2017; Yoshiyama, 2016). Interestingly, SOG1 exists usually in cytoplasm, and can enter the nuclei to regulate hundreds of genes' expression when SOG1 was phosphorylated via MAPK signal pathway, which is dependent on c-abl and MLH1 (Kim et al., 2007; Opdenakker et al., 2012). Thus, unravelling the possible roles of MMR proteins in maintenance and resolution of the G2/M checkpoint and the subsequent mitosis after recovery from DNA lesions in Arabidopsis and other plants under Cd and other stresses could prove interesting and fruitful.

Conclusions

This study indicated that Cd stress induced DNA lesions and G2/M arrest in Arabidopsis roots, which was mediated by *MSH*2 and *MSH*6 genes, but not *MLH*1, of the MMR system via altered expression of G2/M regulatory genes, including *ATM, ATR*, *SOG*1, *WEE*1, *CYCD*4;1, *MAD*2, *CDKA*;1, *CYCB*1;2 and *CYCB*1;1. To our knowledge, this is the first study showing that MSH2 and MSH6 are implicated in G2/M phase arrest triggered by Cd stress in Arabidopsis roots. In addition, endoreduplication occurred mainly from impairment of MSH2 and MSH6, and was not seen in MLH1-deficienct Arabidopsis

roots in response to Cd stress. Moreover, we observed that MSH2 and MSH6 could act as direct sensors of Cd-induced DNA lesions in Arabidopsis plantlets. Because Cd pollution is ubiquitously present in the soil and water, these results provide new insight into the essential mechanisms of *MLH*1, *MSH*2 and *MSH*6 in the G2/M phase arrest induced by DNA damage under Cd stress in other plant seedlings.

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HIGHLIGHTS

- Cd-caused endoreplication occurred mainly by impairment of MSH2 and MSH6 in roots.
- Cd-induced G2 phase arrest was markedly reduced in the MSH2- and MSH6-deficiency.
- Cd-triggered endoreplication was eliminated in MLH1-deficienct Arabidopsis roots.
- MSH2-ATR/ATM is the major signaling cascade in Cd-governed DDR in Arabidopsis.
- MSH2 and MSH6 can act as direct sensors of Cd-induced DNA damage in roots.